STUDY OF PLASMA NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN AS AN EARLY BIO-MARKER OF ACUTE KIDNEY INJURY IN SNAKE BITE

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This Dissertation entitled '**STUDY OF PLASMA NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN AS AN EARLY BIO-MARKER OF ACUTE KIDNEY INJURY IN SNAKE BITE**' is submitted to **The Tamilnadu Dr. M.G.R Medical University, Chennai.** in Partial fulfillment of Regulations for the Award of **M.D Degree** in Biochemistry in the Examinations to be held during April 2011.

This dissertation is a record of fresh work done by the candidate **Dr. R.THAMARAI** during the course of the study (2008-2011). This work was carried out by the candidate herself under my supervision.

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ABBREVIATION

AKI	Acute Kidney Injury
NGAL	Neutrophil gelatinase associated lipocalin
KDa	Kilo Dalton
ELISA	Enzyme Linked Immunosorbent Assay
LDH	Lactate Dehydrogenase
$CaCl_2$	Calcium chloride
ATN	Acute Tubular Necrosis
GFR	Glomerular Filtration Rate
RVV	Russell's Viper Venom
DIC	Disseminated Intravascular coagulation
aPTT	Activated Partial Thromboplastin Time
RBCs	Red Blood Cells
USG	UltraSonoGram
NaOH	Sodium Hydroxide
AUC	Area under curve
ROC	Receiver operating curve
EDTA	Ethylene diamine tetra acetic acid
BT	Bleeding Time
CT	Clotting Time
HRP	Horse Radish Peroxidase
TMB	Tetra Methyl Benzidine
Conc.	Concentration

INTRODUCTION

Acute kidney injury is a clinical syndrome characterized by an abrupt decline in Glomerular filtration rate sufficient to decrease their elimination of nitrogenous waste products and other uremic toxins from the body.[1]

Acute kidney injury is seen in majority of the cases of snake bite. The gravity, spectrum and the outcome varies. Snake bites cause substantial mortality and morbidity in India.

Snakes are fascinating part of nature. Their colour, movement and secret habits make them more mysterious. India is home to some of the most poisonous snakes in the world, most of them are found in rural areas. A large proportion of snake bites occur when people are working barefoot in the field, or while walking at night or early morning through fields or along roads.[2]

Of 3000 species of snakes known to world, in India, we have around 216 species, out of which 52 are known to be poisonous.[3] Majority of bites and consequent mortality is attributable to 5 species viz,

Ophiophagus hannah (king cobra), *Naja Naja* (common cobra), *Daboia rusellii* (Russell's viper), *Bungarus caeruleus* (krait) and *Echis carinatae* (saw scaled viper).[3]

AKI due to snake bite represents a frequent and devastating problem, so early diagnosis can prevent morbidity and mortality. AKI is usually asymptomatic and diagnosed by biochemical monitoring of increase in serum creatinine concentrations.[4]

The rise in serum creatinine is delayed by few days, because the serum creatinine concentration does not increase until half of the kidney function is lost.[5]

In general, there are several non-renal factors influencing the serum creatinine concentration such as body weight, muscle mass, race, age, gender, total body volume, drugs, muscle metabolism and protein intake.[6]

In the cellular insult like AKI, the injury begins by inducing molecular modifications, later evolving into cellular damage. The cells start producing marker of injury and the clinical symptoms develops

subsequently. Thus the detection of biomarkers may provide the much needed window of opportunity for early intervention.

Human Neutrophil Gelatinase Associated Lipocalin is 25kDa secretory glycoprotein belongs to the lipocalin family[7] expressed by kidney cells and undergo an early dramatic upregulation in response to nephrotoxic injury like snake bite.

NGAL is easily measured, unaffected by other biological variables and capable of both early detection and risk stratification[7]. Hence, it would represent a tremendous advance in clinical medicine. NGAL has been found to be very useful for the detection of acute kidney injury within 2 hours of nephrotoxic insult.[7]

AIM OF THE STUDY

1. To estimate plasma neutrophil gelatinase-associated lipocalin (NGAL) in patients with snake bite .
2. To correlate NGAL values with serum creatinine and blood urea and to prove the use of NGAL as an early biomarker of Acute Kidney Injury in patients with snake bite.

REVIEW OF LITERATURE

Acute Kidney Injury is characterized by deterioration in the Glomerular Filtration Rate (GFR) over a period of hours or days resulting in accumulation of nitrogenous waste products.

EPIDEMIOLOGY OF VENOMOUS SNAKES AND SNAKE BITE INDUCED ACUTE KIDNEY INJURY:

Snake bite poisoning is a preventable health hazard in tropics. Very extensive toxicological research is still going on because of high incidence. It accounts for 100-150 deaths per day in India and the annual deaths per year is around 30,000.[3] It is a cause of major preventable death. In India, the highest incidence are seen in Tamil Nadu, West Bengal, Maharastra, Uttar Pradesh and Kerala.[8]

Snake bites happen when the farmers work in field bare footed, unintentionally in a handful of foliage, rolling over the snake while asleep, while working in other plantation and in snake handling[9]. Males are bitten most often than females[9] with majority of bite being on the lower extremities.[10]

Majority of victim are treated initially by traditional snake bite healers. Death often occurs even before the patient reaches hospital[11]. The use of protective foot wear, long trousers and lighting at night could reduce the incidence of snake bites.[12]

Poisonous snakes prevalent in India belong to 3 families[13].

They are:

1) Elapidae: includes Cobras and Krait- Neurotoxic. Renal involvement is less common in victims bitten from members of this family.
2) Viperidae: Russell viper and saw scaled viper- Vasculotoxic.
3) Hydrophidae: Sea snake - Myotoxic.

Renal involvement has been associated with bites from Viperidae[18] and sea snake.[14, 15]

COBRAS:

The two species of Cobras found in India[16] are Common Cobra (Nalla Pambu) and King Cobra (Raja Nagam, Karu Nagam).[17] The Cobra has hood which is on the dorsal side often bears a double or single spectacle mark. It is distributed throughout India. King Cobra is black in

colour and has hood, but no mark on it. They are found in Himalayas, Bengal, Assam and Andaman Islands.

KRAIT:

The two species of krait commonly found in India[16] are common Krait (kattu Viriyan) and Banded Krait (Pattai Kattu Viriyan).[17] The common krait has steel blue or black with white bars on the back. It is distributed throughout India. Banded krait is jet black with yellow stripes on its back. They found in Bengal, Assam, Bihar, Orissa, Madhya Pradesh, Andhra Pradesh and Uttar Pradesh.[17]

VIPER:

The two species of viper that are commonly found in India [16] are Russell's viper (Kannadi Viriyan) and saw scale viper (Surruttai Viriyan).[17] Russell's viper has a triangular head with V shaped mark pointing forwards. It has a white body with dark semilunar spots. It is seen in Maharastra, Punjab, Rajasthan, Tamil Nadu and Andhra Pradesh. Saw scaled viper has many white lines on each flank of the back, with diamond shaped areas between the two lines. It has white mark resembling an arrow over the head. It is seen in hills and plains throughout India.

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SNAKE VENOM:

The venom is a modified salivary secretion. The normal function of snake venom is to immobilize the prey and assist in digestion. The toxic component of snake venom is classified into 4 broad categories namely enzymes, polypeptides, glycoproteins and compounds of low molecular weight. They can also be classified as protein (90-95%) and non protein (5-10%) compounds.[19] It also contains Clostridia, Anaerobes and Gram negative bacilli.

Snake	Fatal Dose for humans	Average delivered dose per bite	Average Fatal Period
Indian Cobra	12mg	0.2g	8h
Common Krait	6mg	0.22g	18h
Russell's Viper	15mg	0.15g	3days
Saw – Scaled Viper	8mg	0.13g	41days

TABLE : Compounds present in Snake Venom [61]	
Enzymes	Phospholipids A_2 (Lecithinase), 5' nucleotidase, collagenase, L-amino acid oxidase, proteinases, hyualuronidase, acetylcholine esterase, phospholipids B, endopeptidase, Kininogenase, factor X, prothrombin activating enzyme.
Non enzyme polypeptides	Polysynaptic (a) neurotoxin (α bungarotoxin and cobrotoxin), presynaptic (b) neurotoxin (β bungarotoxin, crotoxin, and taipoxin) cardiotoxin, crotamine.
Peptides	Pyroglutamyl Peptide
Nucleosides	Adenosine, guanosine, inosine
Lipids	Phospholipids, cholesterol
Amines	Histamine, Serotonin, Spermine
Metals	Copper, Zinc, Sodium, Magnesium

ENZYMES: [61]

SL.NO	Enzymes	Bio- chemical Actions
1	Acetylcholine Esterase	Catalysis and Hydrolysis of Acetylcholine
2	Arginine ester hydrolase	Bradykinin release, interference with clotting
3	Hyaluronidase A	Reduction of Collagen Viscosity
4	Phospholipase A	Un coupling of Oxidative phosphorylation
5	Phospholipase B	Hydrolysis of Lysophosphatides
6	Phophodiesterase	Inhibition of DNA, RNA, Arabinose derivatives
7	5’ Nucleotidase	Specific Hydrolysis of PO_4 Monoesterase which links with 5' position of DNA, RNA
8	L Amino acid Oxidase	Catalysis of Amino Acid
9	Thrombin like enzymes	Depression of Fibrinogen levels
10	Proteolytic enzymes	Tissue destruction and bleeding
11	Collagenases	Collagen digestion

Arginine Esterase:

This enzyme is produced by snakes belonging to Crotalidae and viperidae. This enzyme has action similar to thrombin thereby causes coagulation and it releases bradykinin.

Phospholipase A:

It has a direct lytic effect, hemolytic effect and hydrolysis of phospholipids of RBC membrane, thereby causing sudden fall in Blood Pressure.[13]

Proteinase:

Markedly present in Viper, Crotalidae. It causes tissue changes and destruction.

Anti coagulant effect:

It has anti coagulant effect due to proteolytic disintegration of Fibrinogen.

Coagulant effect:

It has Coagulant effect by converting Prothrombin into Thrombin.

Non Enzymatic Components:

Haemorrhagins (HR-I, HR-II) has direct action on endothelium with Procoagulant and Anticoagulant effects. It causes rapid haemorrhage into visceral organs, vasoconstriction followed by vasodilatation of microvessels, haemorrhages into capillary bed and endothelial destruction.[5]

PATHOGENESIS OF ACUTE KIDNEY INJURY:

The factors that contribute AKI are Direct cytotoxicity, Bleeding, Hypotension, Circulatory collapse, Intravascular hemolysis, Disseminated Intravascular Coagulation and Micro Angiopathic Hemolytic Anemia (MAHA).[20]

Direct Nephrotoxicity:

E.carinatus venom [21] and the demonstration of venom antigen in human victims of snake bite using Enzyme Linked Immuno Sorbent Assay technique have shown that the venom is excreted in the urine.[22] Urinary beta-N acetylglucosaminidase showed considerable change in patients bitten by Russell's viper, indicating a direct toxic effect of venom on the kidney.[23]

The sub lethal dose of *Russell's* viper or *E.carinatus* venom resulted in hemorrhages in the kidneys and mild acute tubular necrosis in 20 % within 24 hrs of envenomation. However, lethal dose developed acute tubular necrosis and fibrin thrombi in 50-75% of glomeruli.[33]

The strongest evidence supporting that direct nephrotoxicity is a dose dependent decrease in inulin clearance and an increase in fractional excretion of sodium, following *Russell's* viper envenomation.[24]

Russell's viper venom administration induces changes in renal plasma flow, glomerular filtration rate, filtration fraction and tubular reabsorption of sodium are reduced, and fractional excretion of sodium and water showed an increase. Both oliguria and a subsequent polyuric phase occurs.

On morphological analysis, the most prominent structural lesions are observed in the renal cortex.[25,30] Extensive damage and loss of glomerular epithelial cells and endothelium are detected with only the basement membrane remaining unaffected. Ballooning and even rupture of glomerular capillaries could be seen.

Another prominent feature of RVV action is on renal cortex and other renal zones, concerned vessels with muscular walls (arteries, veins,

arterioles, venules). The venom lead to complete lysis of vascular smooth muscle cells leaving behind only the basement membrane. Varying degrees of epithelial injury occurred in all tubular segments.

RVV induces a complete disintegration of confluent mesangial cell layers at lower concentration. However, only extremely high doses of RVV lead to microscopically discernible damage. Thus, a dose dependent toxic effect of RVV directes primarily against glomerular and vascular structures and on mesangial cells.

Myoglobinuria, sepsis and hypersensitivity to venomous or anti-venomous protein also contribute towards renal failure [26]. Myoglobinuria generally occurs following sea snake envenomation, which results from necrosis of striated muscles and muscular paralysis.[27]

HYPOTENSION:

Bleeding either into tissues or externally and loss of plasma into the bitten extremity produce hypotension and circulatory collapse. This is caused by venom metalloproteinases, that degrade basement membrane protein surrounding the vessel wall, leading to loss of integrity.[28] Hypotension is also caused by release of bradykinin.[29]

Additionally, vasodilatation and increased capillary permeability, both as a result of direct and indirect effects of venom, can aggravate the circulatory disturbances of shock [30].*Vipera palestinae* venom is thought to cause shock by depression of the medullary vasomotor centre[31].

Bitis arietans causes hypotension by a combination of myocardial depression, arteriolar vasodilatation and increased vascular permeability. Hypotension and circulatory collapse set in motion a chain of hemodynamic disturbances, which culminate in ischemic AKI.

Intravascular Hemolysis:

Intravascular hemolysis plays a role in the pathogenesis of snake bite induced AKI [32]. Hemolysis results from the action of phospholipase A_2 which is present in all snake venoms, and a basic protein called "direct lytic factor", found in elapid venoms.[14,33, 34.]

PhospholipaseA_2 causes hemolysis by direct hydrolysis of red cell membrane phospholipids or causing indirectly via the production of strong hemolytic lysolecithin from plasma lecithin. Severe hemolysis are shown by increased plasma LDH levels, free hemoglobin and late presence of hemolysed red blood cell casts in renal tubules.

The renal failure following snake bite should be considered as an example of the hemolytic uremic syndrome[35]. Microangiopathic hemolysis as seen in HUS is encountered rarely.

More than 70-80% of patients with snake bite induced renal failure have only acute tubular necrosis and do not exhibit the glomerular and arteriolar changes characteristically associated with the hemolytic uremic syndrome.[36]

Disseminated intravascular coagulation:

The human hemostatic system is regulated via a number of critical interactions involving blood proteins, platelets, endothelial cells, and sub-endothelial structures.

Snake venom proteins and peptides are known to activate or inactivate many of these interactions. Snake venoms from viper families, contain many proteins that interact with members of coagulation cascade and the fibrinolytic pathway.

Russell's viper venom (RVV) contains a factor V activating serine proteinase,[37] which has been separated from a factor X-activating protein, also present in this venom. The enzyme (RVV-V) is a single chain

glycoprotein with a molecular weight of 26,100 possessing one glycosylation site near the carboxy terminus.

RVV-V cleaves a single bond to convert factor V to factor Va (the activated clotting protein). *Russell's* viper venom contains a potent activator of human coagulation factor X; this enzyme is designated as RVV-X. [38] Factor X activators have been isolated from *bathrops atrox* and several other snake species. *Russell's viper* venom activates factor IX by cleavage of a single peptide bond resulting in the formation of factor IXa.

There are several different types of prothrombin activators in snake venom. The activity of members of group I is not influenced by components of prothrombin activator complex (factor Va, $CaCl_2$ and phospholipid).[39] Ecarin, from *E.carinatus* venom, is the most well studied member of this group. Group II activators resemble factor Xa and cleave both peptide bonds in prothrombin, leading to active 2-chain thrombin. Their activity is strongly stimulated by phospholipids and factor Va in the presence of $CaCl_2$.

By contrast activators in group III require only phospholipid and $CaCl_2$ for the activation of prothrombin. They do not require factor Va, but

appear to possess a co-factor that is tightly bound to the catalytic subunit that plays a similar role to factor Va in prothrombin activation.[40]

Although thrombin has many activities, the ability of some snake venom enzymes to clot fibrinogen has resulted in these enzymes being called "thrombin-like"[40,41] .These are widely distributed primarily in the venom of snakes from true vipers (*Bitis gabonica*, *Cerastes vipera*) and pit vipers (*Crotalus adamanteus*, *Bothrops atrox*). Snake venom fibrinogen clotting enzymes have been classified into several groups based on the rates of release of fibrinopeptides A and B from fibrinogen.

One mechanism of the anticoagulant action of snake venom proteins is attributed to the activation of protein C. Activated protein C degrades factors Va and VIIIa and therefore, has anticoagulant activity. Another mechanism of anticoagulation involves inhibition of blood coagulation factors IX and X by venom protein(s) that binds to either or both.

Finally, anticoagulation is also achieved through the action of snake venom phospholipases that degrade phospholipids involved in the formation of complexes critical to the activation of the coagulation pathway.[42]

Direct acting fibrinolytic enzymes have been characterized as zinc metalloproteinases and are classified as either alpha or beta chain fibrinogenases. Snake venom contains a number of platelet active components, including those that cause platelet aggregation and those that inhibit platelet aggregation.[43]

The final coagulation disturbance depends upon the balance among the activity of procoagulant and anticoagulant, fibrinolytic and fibrinogenolytic components of injected venom. Disseminated intravascular coagulation (DIC) is a consistent feature in patients bitten by *Russell's viper*, *E.carinatus*, *boomslang* and *pit vipers*.[44]

The presence of fibrin thrombi in the renal microvasculature and in the glomerular capillaries, and the findings of MAHA and thrombocytopenia due to DIC results in cortical necrosis.[33]

Snake venom initiates a chain reaction involving the coagulation, fibrinolytic, kinin and complement system. Venom induced alteration lead to vascular coagulation and deposition of fibrin thrombi in blood vessels. Intraglomerular fibrin deposition of lesser degree causes ATN via a temporary hemodynamic alteration.

0.4 mg/kg of *Bothrops jararaca* venom produced functional and morphological changes observed in snake-bite induced AKI.[35] There was intravascular hemolysis, as shown by significant decrease in hematocrit, an increase in plasma LDH levels and free hemoglobin.

Light and electron microscopy showed massive fibrin deposition in glomerular capillaries apart from proximal and distal tubular necrosis and red blood cell casts in renal tubules. Ischemia related to glomerular coagulation and intravascular hemolysis are the most important pathogenetic factors causing a decrease in the GFR, although direct venom nephrotoxicity could not be excluded.[35]

PATHOPHYSIOLOGY OF ACUTE TUBULAR NECROSIS:

After nephrotoxic injury to the kidney, an early response is loss of the brush border and polarity of the epithelial cell, with mislocation of adhesion molecules, Na^+, K^+-ATPase and other proteins.[54]

With increasing injury, cell death occurs by means of necrosis or apoptosis. Some of the necrotic debris is released into the lumen, where it interacts with luminal proteins and can ultimately result in obstruction. Because of the mislocation of adhesion molecules, viable epithelial cells lift off the basement membrane and are found in the urine.

The kidney can respond to injury by initiating a repair process if provided sufficient nutrients and sufficient oxygen delivery and if the basement membrane integrity has not been altered irreparably.

Viable epithelial cells migrate and cover denuded areas of the basement membrane. Cells replacing the epithelium may be derived from dedifferentiated epithelial cells or from a subpopulation of progenitor cells in the tubule. The cells then undergo division and replace lost cells. Ultimately the cells differentiate and re-establish the normal polarity of the epithelium.

HISTOLOGY:

Renal histology shows predominantly either acute tubular or cortical necrosis. A number of glomerular changes have been described but their significance is not known.

Acute Tubular Necrosis:

Acute tubular necrosis is the predominant lesion in 70-80% of patients with AKI.[33] On light microscopy, the tubules appear dilated and are lined

by flattened epithelium. Severe cases exhibit cell necrosis and desquamation of necrotic cells from the basement membrane. Hyaline, granular or pigment casts are seen in tubular lumina.

Varying degrees of interstitial edema, hemorrhage and inflammatory cell infiltration are present. Biopsies reveal regenerating tubular epithelium. Intrarenal blood vessels are usually unaffected.

On ultrastructural examination, proximal tubules show dense intra cytoplasmic bodies representing degenerating organelles or protein resorption droplets. Small areas of basement membrane are denuded. Distal tubular cells have a dilated endoplasmic reticulum and many degenerating organelles.

Apoptosis is a prominent feature in the distal tubules, indicating a high cell turnover. In the interstitium, fibroblasts appear active, with increased numbers of organelles and cytoplasmic processes. Mast cells and eosinophils show both granulated and partially degranulated form.[45]

Ultrastructural abnormalities are notable in both large and small caliber vessels.[45] Medullary vessels are severely affected, with markedly swollen, focally necrotic, endothelial cells obliterating the lumen. Smooth

muscle cells show cytoplasmic vacuoles, which are empty or filled with granular material.

The severe vascular lesions, distal tubular apoptosis, and presence of mast cells, eosinophils, and active fibroblasts in the interstitium are observed in acute tubular necrosis of snake bite induced AKI.

Acute Cortical Necrosis:

Bilateral diffuse or patchy cortical necrosis has been observed following bites by E.carinatus. Cortical necrosis appears to be more common among Indian patients.

The presence of fibrin thrombi in the arterioles is a prominent feature in these patients. A narrow subcapsular rim of cortex often escapes necrosis. The area underlying this shows necrosis of glomerular as well as tubular elements. The necrotic zone is often bordered by an area of hyperemia and leukocytic infiltration.

Calcification of necrotic areas may occur at a later stage. Varying numbers of glomeruli are spared with patchy cortical necrosis. With healing, fibroblastic proliferation and organization of thrombi are seen[45].

Renal ultrastructure in cortical necrosis following Russell's viper shows glomeruli with collapsed capillary basement membrane, and denuded foot processes. No viable endothelial or mesangial cell can be identified, but swollen rounded cells, possibly of endothelial origin, are seen in some capillary lumina.

Endothelial swelling of small arterioles and necrosis of peritubular capillaries are also seen. The tubular basement membrane are intact, but the epithelium shows degenerative changes. The urinary space contained unidentified cells with large cytoplasmic vacuoles[45].

The tubular basement membrane is thickened, and the cortical tubules are lined by flattened epithelium, with large nuclei and a dilated endoplasmic reticulum. Fibroblastic proliferation is seen in the interstitium.

Glomerular Lesions:

"Proliferative glomerulonephritis" are reported in patients bitten by *E.carinatus* and also crescentic glomerulonephritis, following puff adder bites, presenting as AKI.[26] Renal lesions of proliferative nephritis with

crescents has developed within 24-48 hours. Crescentic glomerulonephritis are found to occur following *Russell's viper* bite.

Russell's viper bite, shows thickening of the mesangial areas and mild mesangial proliferation and diffuse glomerular hypercellularity (marked mesangial proliferation). Other glomerular changes are ballooning of capillaries, endothelial swelling, mesangiolysis and splitting of the glomerular basement membrane.

Immunofluorescence microscopy shows IgM, C3 and fibrin deposits. Also a diffuse and intense mononuclear cell infiltrate has been noted in the interstitium, suggesting the occurence of an acute interstitial nephritis.[46]

RIFLE and AKIN criteria for diagnosis of AKI

RIFLE criteria

Class	GFR Criteria	Urine output criteria
Risk	Increased S.creatinine x 1.3 or GFR decrease > 25%	Urine output < 0.5 ml/kg/h x 6h
Injury	Increased S.creatinine x 2 or GFR decrease > 50%	Urine output < 0.5 ml/kg/h x 12h
Failure	Increased S.creatinine x 3 or GFR decrease > 75% or S.creatinine ≥ 4 mg/dl (Acute rise of ≥ 0.5 mg/dl)	Urine output < 0.3 ml/kg/h x 24h of Anuria x 12h
Loss	Persistent AKI = complete loss of renal functions > 4 weeks	
ESKD	End stage kidney disease > 3 months	

AKIN classification

Stage	Serum creatinine criteria	Urine output criteria
1.	Increase in serum creatinine of ≥ 0.3 mg/dl Or increase to ≥ 150 to 200% from baseline	Urine output < 0.5 ml/kg/h x 6h
2	Increase in serum creatinine to > 200 to 300% from baseline	Urine output ≤ 0.5 ml/kg/h x 12h
3	Increase in serum creatinine to > 300% from baseline	Urine output < 0.3 ml/kg/h or anuria x 12h

BIOMARKER:

Early detection of renal injury is of key importance to impact morbidity and mortality and the introduction of potential therapeutic agents into the course of renal disease.

Human NGAL:

Human NGAL (synonyms: lipocalin 2, siderocalin, neutrophil lipocalin) is 25kDa secretory glycoprotein belongs to lipocalin superfamily[7]. NGAL has been orginally isolated from the secretory granules of activated human neutrophils.[46]

Human NGAL consists of a single disulfide bridged polypeptide chain of 178 amino acid residues with a calculated molecular mass 22kDa[46] and its glycosylation increases its apparent molecular mass to 25kDa.

They are small secreted proteins characterized by their ability to bind small, hydrophobic molecules in a structurally conserved pocket formed by 8 antiparallel beta pleated sheets, it binds to specific cell surface receptors to form macromolecular complexes.[47]

NGAL is a protease resistant polypeptide, released from the distal tubules, secreted with the urine or backleaking to the plasma, freely filtered, reabsorbed in the proximal tubules through endocytic megalin receptors or finally secreted with the urine.[48] Because of its small molecular size, and resistant to degradation, NGAL is readily excreted.

NGAL is responsible for growth and differentiation of renal tubular epithelial cells and exerts bacteriostatic effects in the distal urogenital tract by interference with bacterial siderophore-mediated iron acquisition. This sidreophore iron complex limits proximal tubular damage and reduces apoptosis.[48]

Gene expression studies in AKI have also shown rapid upregulation of NGAL mRNA in the thick ascending limb of Henle's loop and the collecting ducts, with resultant synthesis of NGAL protein in the distal nephron (the renal pool) and secreted into the urine.

AKI results in increased NGAL mRNA expression in distant organs, especially the liver and spleen, and the over-expressed NGAL protein is most likely released into the circulation and constitutes the systemic pool.

Additional contributions to the systemic pool in AKI may derive from the fact that NGAL is a known acute phase reactant and released from neutrophils, macrophages, and other immune cells.[51] Any decrease in glomerular filtration rate resulting from AKI would be expected to decrease the clearance of NGAL, with further accumulation in the systemic pool. These factors are responsible for the elevated levels of NGAL in the plasma during AKI.[51]

Raised plasma levels of NGAL were found to be strongly correlated with decreased renal function in patients with renal damage due to nephrotoxicity.[49, 50]

Hence, Plasma NGAL has emerged as a biomarker that predict the development of AKI in patients 1-3 days earlier than serum creatinine.[50, 51, 52]

NGAL appears to be an early potential 'real time' biomarker for AKI and its expression is proportional to the severity of renal injury.

MATERIALS AND METHODS

Study Design : Prospective case control study.

Place of study : Department of Bio chemistry.
Thanjavur Medical College and Hospital.

Period of study : October 2009 to July 2010.

Sample size : 50 Cases and 50 Controls.

Sampling procedure :

100 patients admitted for snake bite were followed with estimation of NGAL, serum creatinine, blood urea to diagnose AKI. Patients developed AKI were considered as cases. Patients not developed AKI were considered as Controls.

Inclusion Criteria :

Patients admitted within 12 hours of snake bite.

Exclusion Criteria :

1. Snake bite patients with elevated serum creatinine on admission.
2. Known Hypertensive on treatment.
3. Chronic Kidney Disease.
4. Known Diabetic on treatment.
5. Systemic and Urinary tract infections.
6. Past history of renal disease.

STUDY PROTOCOL

History:

A detailed history was elicited for

- Co-morbid diseases and concomitant drugs intake.
- Species of snake.
- Site and time of bite.
- Native treatment.
- Treatment before hospitalization.
- Hematuria, hemetemesis, hemoptysis, bleeding gums and bleeding from the site of bite.
- History of reduced urine output, oliguria and anuria.

CLINICAL EXAMINATION: A thorough physical examination was done to look for local and systemic features of envenomation like fang marks, cellulitis, bleeding from the site of bite, local necrosis, blistering, gangrene, regional lymph node enlargement and features of gum bleeding, epistaxis, ecchymosis.

All vital signs were looked for.

Features of uremic symptoms were looked for.

INVESTIGATIONS:

1. Complete Blood Count
2. Bleeding time
3. Clotting time
4. Urine Albumin, Sugar, Deposits including pus cells, RBCs.
5. Random blood sugar done by Glucose Oxidase -Peroxidase Method.
6. USG Abdomen done to exclude Chronic kidney disease.
7. Plasma NGAL
8. Blood urea and serum creatinine
9. Serum electrolytes

COLLECTION OF SPECIMENS

Blood samples were collected under aseptic precautions in EDTA coated polypropylene tubes. Blood was centrifuged and plasma was separated. The specimens were freezed at -20°C for storage.

One part of the blood was transferred to plain tubes and the sample was centrifuged and serum separated for the analysis of blood urea, serum creatinine, and serum electrolytes.

ESTIMATION OF NGAL IN PLASMA:

METHODOLOGY:

Solid phase enzyme linked immunosorbent assay.

PRINCIPLE:

The micro wells are coated with monoclonal antibody against human NGAL. Aliquots of calibrators, diluted samples and controls are incubated with HRP conjugated NGAL antibody. NGAL bound to antibody, while unbound materials are washed off. Upon addition of the chromogen substrate, the HRP linked to the bound detection antibody reacts with the substrate to generate a colored product. The color intensity is read at 450 nm in an ELISA reader and the intensity of the color developed is proportional to the concentration of NGAL.

KIT CONTENTS:

1. 12 × 8 coated Microwells (96 wells) +Frame
2. 5 × Sample diluents Conc. (1 × 60 mL)
3. NGAL Rapid calibrator 1-6 (6 × 1 mL)
4. 25 × Wash solution conc. (1× 30 mL)
5. Horse Radish Peroxidase-conjugated NGAL Antibody (1 × 6 mL)
6. Tetra Methyl Benzidine (TMB) substrate (1 × 12 mL)
7. Stop solution: 0.5 mol / L sulphuric acid (1 × 16 mL)
8. Polypropylene U –Microwell Plate 96 wells

NGAL Calibrator Concentrations	ng/mL
Calibrator 1	0
Calibrator 2	0.3
Calibrator 3	2.2
Calibrator 4	5.1
Calibrator 5	10.6
Calibrator 6	19.2

PREPARATION OF REAGENTS:

1. All specimens and reagents are brought to room temperature (20-25°C). Specimens are mixed thoroughly by gentle inversion and visible particulate matter is cleared by low-speed centrifugation.
2. Wash Solution: 25x Wash Solution Concentrate is diluted by pouring the total contents of the bottle (30 ml) into 1-L graduated cylinder and deionized water is added to make a final volume of 750 ml. It is thoroughly mixed.
3. Sample Diluent: 5x Sample Diluent Concentrate (contains yellow dye to aid pipetting) is diluted by pouring the total contents of the bottle (60 ml) into a 500-ml graduated cylinder and deionized water is added to make a final volume of 300 ml. It is thoroughly mixed.
4. NGAL Rapid Calibrators (ready to use): It is not diluted further.
5. HRP- conjugated NGAL Antibody: It is not diluted further.

6. Stop Solution: It is not diluted further.
7. TMB Substrate: It is not diluted further.

ASSAY PROCEDURE:

1. The appropriate wells for setting up of calibrators, diluted patients specimens and diluted controls are assigned
2. Samples are diluted according to the expected NGAL concentrations, 1/100 for plasma.
3. A sufficient volume of each calibrator, each diluted sample and diluted controls are pipette into the appropriate wells of the polypropylene U-microwell plate to permit subsequent transfer of 50 µL volumes to corresponding coated microwell.
4. 50 µL volumes of HRP – conjugated NGAL antibody are pipetted into the coated microwells. Then with a multichannel pipette, 50 µL volumes of calibrator solutions, diluted samples from the U wells are rapidly transferred into the corresponding coated wells already containing the detection antibody.
5. The wells are covered and incubated for 30 minutes at room temperature on a shaking platform and set at 200 per minute.
6. The contents of the microwells are aspirated and washed with 300 µL of diluted wash solution to facilitate removal of unbound material.

7. 100 µL of TMB substrate are dispensed into each microwell and incubated for 15 minutes at room temperature in the dark.
8. 100 µL of Stop solution is added to each well and mixed by gentle shaking for 20 seconds. The wells are read within 30 minutes.
9. The absorbances of the wells are read at 450nm in an ELISA reader.

CALCULATION OF RESULTS:

Calibration graph:

Calibration curve was constructed by plotting the mean of duplicate absorbance values for each NGAL Calibrator on the y-axis against the corresponding NGAL concentrations in ng/mL on the x-axis.

NGAL values of subjects:

The NGAL concentration of each diluted sample was found by locating the point on the curve corresponding to the mean of duplicate absorbance values for the diluted samples and reading its corresponding concentrations in ng/mL from the x-axis.

The concentration of NGAL in diluted specimens was calculated by multiplying the result by the sample dilution factor.

Storage:

All reagents are stored at 2-8°C.

Reference value:

33-106 ng/mL.

QUANTITATIVE DETERMINATION OF SERUM CREATININE

[MODIFIED JAFFE'S KINETIC METHOD]

PRINCIPLE OF THE METHOD:

Creatinine present in the sample reacts with picric acid in alkaline medium forming creatinine picrate (red coloured complex) which is measured at 500 nm.

REAGENTS:

Reagent 1 (picrate):

Picric acid	34.9 mmol / L
Sodium Hydroxide	45 mmol / L

Reagent 2 (Sodium Hydroxide):

Sodium hydroxide	0.26 mol/L

Standard (Creatinine 2 mg/dL):

Creatinine	0.020 g/L

STORAGE: 2ºC-8ºC

PREPARATION OF WORKING SOLUTION:

The reagents are allowed to attain room temperature . Equal volumes of reagent 1 & reagent 2 are mixed in a clean beaker.

PROCEDURE:

The sample and the working solution are brought to room temperature prior to use.

GENERAL SYSTEM PARAMETERS:

Reaction Type	:	Fixed Time
Reaction slope	:	Increasing
Wavelength	:	500 nm (490-510 nm)
Flowcell Temp.	:	25°C, 30°C or 37°C
Delay Time	:	30 seconds.
No. of Readings	:	120 seconds.
Sample Volume	:	100 µL
Reagent Volume	:	1.0 mL
Pathlength	:	1 cm
Std. Concentration	:	2 mg/dL
Zero Setting With	:	Distilled water

The instrument is set with using above system parameters.

PROCEDURE:

3 test tubes are taken and labeled them as blank (B), Standard (S), Test (T). 1 ml of working reagent is added to 3 test tubes. 100µL of sample is added to test tube labeled 'T' and 100µL of standard is added to test tube labeled 'S'. It is mixed and read immediately.

	Blank	**Standard**	**Test**
Reagent	1 mL	1 mL	1 mL
Standard	--	100 µL	--
Sample	--	--	100µL

LINEARITY:

This method is linear upto 10 mg/dL.

REFERENCE VALUES:

Serum Creatinine:

Males : 0.6-1.3 mg/dL

Females : 0.6-1.1 mg/dL

QUANTITATIVE ESTIMATION OF UREA BY UREASE METHOD (BERTHELOT'S REACTION):

PRINCIPLE:

Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in the presence of hypochlorite to form an indophenol which with alkali gives a blue coloured compound. The intensity of the colour is proportional to the concentration of urea in the sample and is measured at 546nm.

REAGENTS:

Reagent 1(Urease):

Urease	> 1KSU/L

Reagent 1A (Buffer):

Disodium EDTA	0.1 mol/L
Sodium Nitroprusside	6 mmol/L

Reagent 2 (Phenol):

Phenol	1.8 mmol/L

Reagent 3 (Hypochlorite):

Sodium hypochlorite	0.47 mol/L

Standard (Urea 40 mg/dL):

Urea	0.4 g/L

REAGENT RECONSTITUTION

SOLUTION (1)

The contents of one bottle of reagent 1A is transferred into one bottle of reagent 1 and mixed gently.

SOLUTION (2)

77 mL of distilled water is added into one bottle of reagent and mixed gently.

SOLUTION (3)

77 mL of distilled water is added into one bottle of reagent 3and mixed gently.

PROCEDURE

The samples and the reconstituted solutions are brought to the room temperature prior to use.

General System Parameters:

Reaction Type	:	Endpoint
Reaction Slope	:	Increasing
Wavelength	:	546 nm (530 -570 nm)
Flow cell Temperature	:	30 º C
Incubation	:	10 minutes (1 st step) 15 minutes (2 nd step) at 37 ºC
Sample Volume	:	10 µL
Reagent Volume	:	3.1 mL (Reagents 1+2+3)
Standard Concentration	:	40 mg/ dL
Zero Setting With	:	Reagent Blank

The instrument is set using above system parameters.

PROCEDURE:

3 test tubes are taken and labeled them as blank(B), Standard(S),Test(T). 100µL of solution 1 is added to 3 test tubes. 10µL of sample is added to test tube labeled 'T' and 10µL of standard is added to test tube labeled 'S'.

	Blank	**Standard**	**Test**
Solution 1	100 µL	100µL	100µL
Standard	-	10 µL	-
Sample	-	-	10µL

Incubated for 10 minutes at 37°C.It is mixed and then added

Solution 2	1.5 mL	1.5 mL	1.5 mL
Solution 3	1.5 mL	1.5 mL	1.5 mL

Incubated for 15 minutes at 37°C.It is mixed and then read.

LINEARITY

The method is linear upto 200 mg/dL

REFERENCE VALUE: 10-50 mg/dL

RESULTS AND STATISTICAL ANALYSIS

Table 1 : Age distribution among control and case group

Age Group	Control		Case	
	No.	%	No.	%
Upto 30 yrs	12	24	14	28
31-40	12	24	10	20
41-50	16	32	16	32
51 & above	10	20	10	20
Total	50	100	50	100
Range Mean S.D.	20 - 58 yrs 39.5 yrs 11.1 yrs		20 - 60 yrs 39 yrs 11.2 yrs	
'p'	0.8119 Not Significant			

Table 2 : Sex Distribution among control and case group

Sex	Control		Case	
	No.	**%**	**No.**	**%**
Male	30	60	33	66
Female	20	40	17	34
Total	50	50	50	100
'p'	0.6787 Not Significant			

Table 3 : Comparison of NGAL values in the control and case group

NGAL values	Control	Case
Range	14 – 54	38-1020
Mean	33.9	536.7
S.D.	9.2	244.7
'p'	**0.0001 Significant**	

Table 4 : Comparison of NGAL values in different age groups among control and case group

Age group	Control		Case	
	Mean	**S.D.**	**Mean**	**S.D.**
Upto 30 yrs	33.0	6.6	497.1	253.4
31-40	31.3	11.1	403.5	243.2
41-50	38.3	8.2	662	223.7
51 & above	30.4	9.3	525.1	201.2
'p'	0.0953 Not significant		0.0561 Not significant	

Table 5 : Comparison of NGAL values among males and females in control and case group

Sex	Control		Case	
	Mean	**S.D.**	**Mean**	**S.D.**
Male	36.7	9.1	555.7	224.3
Female	32.6	7.8	500.0	283.8
'p'	0.6671 Not significant		0.6671 Not significant	

Table 6 :Comparison of Creatinine values in control and case group

Creatinine values on	Control		Case		'p'
	Mean	**S.D.**	**Mean**	**S.D.**	
Day 1	0.9	0.14	0.89	0.12	0.6446 Not significant
Day 2	0.99	0.1	0.98	0.1	0.4983 Not significant
Day $\geq$ 3	0.95	0.13	3.12	0.45	**0.0001** **Significant**

Table 7 :Comparison of Urea values in Control and Case Group

Urea values on	Control		Case		'p'
	Mean	**S.D.**	**Mean**	**S.D.**	
Day 1	35.7	3.7	36.3	3.3	0.5466 Not significant
Day 2	36.3	3.8	37.4	3.0	0.0724 Not significant
Day $\geq$ 3	34.1	5.6	71.12	17.9	**0.0001** **Significant**

Table 8 : Comparison of NGAL, with serum creatinine and blood urea on Day 1

Day 1		Mean	S.D	'p'
NGAL	Cases Controls	536.7 33.9	244.7 9.2	0.0001
Serum creatinine	Cases Controls	0.89 0.9	0.12 0.14	0.6446 Not significant
Blood urea	Cases Controls	36.3 35.7	3.3 3.7	0.5466 Not significant

Table 9 : Pearson's Correlation Coefficient between NGAL, Creatinine and Urea

Correlation Coefficent between	**Correlation Coefficient**	**Correlation**
NGAL & Creatinine on Day 1	0.0494	Not Correlated
NGAL & Creatinine on Day 2	0.0569	Not Correlated
NGAL & Creatinine on Day≥ 3	**0.5432**	**Correlated**
NGAL & Urea on Day 1	0.1318	Not Correlated
NGAL & Urea on Day 2	0.0961	Not Correlated
NGAL & Urea on Day≥ 3	**0.5035**	**Correlated**

RESULTS

- Table 1 shows age distribution among case and control subjects. There is no significant age differences between the two groups.

- Table 2 shows sex distribution between cases and controls. There is no significant differences between the two groups.

- Table 3 shows the plasma levels of NGAL between cases and controls. Plasma NGAL level is significantly higher in cases with mean of 536.7 than control group with mean of 33.9. This difference in the plasma NGAL level between cases and control gives a 'p' value of 0.0001 which is highly significant.

- Table 4 shows age matched Plasma NGAL values between cases and controls. There is no significant difference in plasma NGAL value in different age groups among cases with 'p' value 0.0561 and controls with ‘p’ value 0.0953.

- Table 5 shows the plasma NGAL values among male and female groups in cases and controls. There is no significant difference in Plasma NGAL values between males and females in cases with 'p' value of 0.6671 and controls with 'p' value of 0.6671.

- Table 6 shows serum creatinine values on day 1, day 2, day 3 and above between cases and controls. There is no significant change in serum creatinine level on day1 and day 2 between cases and controls. On day 3 and above serum creatinine is elevated in cases than in controls with 'p' value of 0.0001 which is significant.

- Table 7 shows blood urea values on day1, day2, day 3and above between cases and controls. Blood urea is not significantly elevated on day1 and day2 between cases and controls. On day 3 and above, blood urea is elevated in cases than controls with 'p' value of 0.0001.

- Table 8 shows comparison of NGAL values, serum creatinine and blood urea between cases and controls on Day1. There is a significant rise in the NGAL levels among cases than controls with ‘p’ value 0.0001. There is no significant rise in serum creatinine and blood urea between cases and controls.

DISCUSSION

This study evaluates the use of NGAL for early diagnosis of AKI than serum creatinine and blood urea because NGAL raises in plasma even before renal cell necrosis occurs after the nephrotoxic insult like snake bite. AKI is an important complication of snake bite and a major cause of morbidity and mortality in India.

The traditional laboratory test to diagnose AKI such as estimation of serum creatinine will be detected in serum only after 50% of renal cell death has occurred. Serum creatinine does not accurately depict kidney function until a steady state has been reached, which requires more than 3 days.[1,51]

Hence it does not allow for early detection of acute renal injury. Damage to renal tubules alone is not sufficient to result in a change in a parameter of kidney function such as serum creatinine.

The change in serum creatinine, however, does not discriminate the time and type of renal insult or the site and extent of glomerular or tubular injury. Levels are relatively insensitive to small changes in GFR and may lag behind changes in GFR by several days.

In addition, in cases of more extensive tubular injury, there is a lag in time between the injury and an increase in serum creatinine. Sensitive biologic markers for renal tubular injury are needed in order to detect early kidney injury.

In the present study, the mean value of Plasma NGAL is 536.7 in the case group shows a significant rise than the control group with the mean of 33.9 during the initial hours of snake bite with 'p' value 0.0001.

The values of plasma NGAL and serum creatinine in cases and controls are compared, it shows a marked elevation of plasma NGAL than serum creatinine during the early hours of snake bite with 'p' value of NGAL <0.0001 and 'p' value of serum creatinine 0.6446.

Those patients who showed elevated NGAL level on initial hours of admission with Mean of 244.7 is found to have developed Acute Kidney Injury on serial estimation of serum creatinine for 3 days and above.

This study shows the rise in serum creatinine and blood urea are observed only on the 3rd day of nephrotoxic insult who developed acute kidney injury.

AREA UNDER THE CURVE

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.992	.006	.000	.000	1.000

With an area under the curve of 0.992 and a standard error of 0.006, NGAL shows an asymptotic significance of 0.000 proving it as a better predictor over creatinine, in diagnosing AKI. This suggest utility of NGAL as an excellent standalone marker.

This study correlates with the reported AUC in the 0.93-1 range given in previous studies[53] done by Mishra et al, Benett et al.

Various epidemiological and clinical studies have shown strong association between rise in NGAL and early detection of AKI than serum creatinine which is delayed by several days.

In the Pearson's correlation analysis, as shown in table 9, there is a no significant correlation noticed between rise in plasma NGAL and serum

creatinine on the day of admission. Progressive kidney damage leading to rise in serum creatinine shows a significant correlation between NGAL and creatinine on day 3.

Our results clearly indicate that plasma NGAL is a powerful early biomarker of AKI that precedes the increase in serum creatinine by 3 days.

Therefore it is necessary to detect acute tubular injury in a timely manner with the use of NGAL, so that early intervention can be initiated.

CONCLUSION

This study on snake bite patients shows that plasma NGAL is a promising early predictive bio-marker of acute kidney injury than blood urea and serum creatinine. This novel bio- marker might facilitate earlier diagnosis of acute kidney injury and management decision including administration of specific preventive and therapeutic strategies, potentially resulting in fewer morbidity and mortality.

This makes NGAL potentially a very sensitive marker of different degrees of renal injury.

SCOPE FOR FUTURE STUDY

In addition to early diagnosis and prediction, it would be desirable to use NGAL capable of predicting clinical outcomes, allowing for risk stratification and monitoring response to therapeutic interventions.

Master chart

CASES

SL. No	Age	Sex	Types of Snake	Plasma NGAL ng/mL	Sr. Creatinine [mg/dL]			Blood Urea [mg/dL]			Electrolytes mEq/L		BT mt	CT mt	Urine Examination	
					Day 1	Day 2	Day ≥3	Day 1	Day 2	Day ≥3	Na	K			Alb	RBC
1.	20	M	viper	440	0.8	0.9	2.4	28	30	60	140	4.0	3	10	+	Nil
2.	26	M	unknown	483	0.7	0.9	2.3	36	34	57	135	3.8	4	10	+	Nil
3.	29	F	viper	107	1.0	1.0	2.9	39	40	61	142	4.2	4	8	+	1-2
4.	20	F	viper	706	0.8	1.0	3.2	33	36	98	145	4.4	3	7	++	0-1
5.	39	M	viper	662	1.1	1.1	2.7	39	40	74	138	5.0	4	9	+	2-3
6.	42	F	unknown	702	0.9	0.9	3.8	40	41	106	142	5.0	3	8	++	1-2
7.	40	M	viper	685	0.7	1.1	3.7	39	41	57	136	3.8	3	12	++	1-2
8.	44	M	viper	107	0.9	1.1	3.4	38	39	60	140	3.6	5	10	+	Nil
9.	47	M	viper	717	1.1	1.0	3.2	39	39	63	144	4.0	4	7	+	0-1
10.	49	M	unknown	588	0.8	0.9	3.5	38	40	62	138	4.2	3	9	+	1-2
11.	42	M	viper	708	0.8	1.0	3.7	35	36	104	146	4.8	3	8	++	0-1
12.	54	M	viper	470	0.8	0.8	3.4	40	40	74	138	3.8	4	12	+	Nil
13.	43	M	viper	637	1.0	1.0	3.9	37	39	53	136	3.5	3	7	+	1-2
14.	33	F	viper	99	0.9	0.9	3.4	34	34	63	142	4.2	3	9	+	2-3
15.	24	M	unknown	476	0.9	1.1	2.2	32	33	53	144	3.8	3	8	+	1-2
16.	21	M	viper	748	0.8	1.0	2.6	38	38	87	148	3.7	4	12	+	2-3
17.	38	M	viper	476	1.0	1.1	2.2	34	36	57	150	4.8	3	7	+	2-3
18.	33	M	viper	126	0.9	0.9	2.9	39	40	50	138	5.0	3	9	+	1-2
19.	32	F	viper	500	1.0	1.0	2.8	35	37	68	142	4.1	4	9	+	Nil
20.	32	F	viper	588	0.9	1.0	2.9	35	35	56	141	3.8	3	8	+	1-2
21.	30	F	unknown	621	0.8	0.9	3.0	38	38	72	140	4.1	4	7	++	1-2
22.	33	F	viper	520	0.9	0.9	3.2	34	37	63	135	3.8	5	7	++	2-3
23.	31	F	viper	110	0.9	1.1	3.1	39	39	75	138	4.0	3	10	+	1-2
24.	46	F	Viper	629	0.8	0.9	3.2	36	39	62	136	4.2	4	8	+	Nil

SL. No	Age	Sex	Types of Snake	Plasma NGAL ng/mL	Sr. Creatinine [mg/dL]			Blood Urea [mg/dL]			Electrolytes meq/L		BT mt	CT mt	Urine Examination	
					Day 1	Day 2	Day ≥3	Day 1	Day 2	Day ≥3	Na	K			A1b	RBC
25.	48	F	Viper	570	1.0	1.0	2.9	35	35	55	148	3.6	3	10	+	1-2
26.	47	F	unknown	532	0.9	0.9	3.5	37	39	54	142	4.0	4	8	++	Nil
27.	46	M	viper	613	0.9	1.1	2.8	35	36	58	150	3.5	3	7	+	2-3
28.	43	M	viper	527	0.9	1.0	3.0	41	42	76	141	3.9	5	8	+	1-2
29.	21	M	viper	385	1.0	1.1	2.3	34	37	64	144	4.2	3	10	Nil	2-3
30.	22	M	unknown	380	0.8	1.1	3.0	34	34	58	142	4.5	4	11	+	1-2
31.	30	M	viper	515	0.8	0.9	3.2	38	38	51	138	3.8	3	8	++	2-3
32.	24	M	viper	444	0.6	0.8	2.6	35	37	82	137	4.0	3	10	+	Nil
33.	37	M	unknown	104	0.9	1.1	2.8	36	38	50	143	3.5	3	9	Nil	1-2
34.	25	M	viper	94	0.9	0.9	3.0	40	44	60	148	4.4	4	8	Trace	1-2
35.	25	F	viper	610	0.9	0.9	3.1	28	29	106	141	4.7	3	10	+	2-3
36.	29	M	viper	578	1.0	1.0	2.8	29	32	56	136	3.8	5	12	+	1-2
37.	46	M	viper	710	0.8	1.1	2.8	35	38	102	135	3.6	4	10	+	2-3
38.	42	M	unknown	577	1.0	1.1	3.0	36	39	89	147	4.2	4	8	++	1-2
39.	52	M	viper	682	1.1	1.1	3.5	37	37	73	141	4.4	3	8	+	1-2
40.	60	M	viper	629	0.9	0.9	3.4	35	37	84	138	4.7	4	9	Nil	2-3
41.	42	M	viper	802	0.8	1.0	3.5	40	40	57	142	3.6	3	10	+	1-2
42.	55	M	viper	528	1.1	1.1	3.5	32	39	86	137	4.2	4	9	Trace	Nil
43.	47	M	viper	625	0.9	0.9	3.1	34	35	103	144	3.9	3	11	+	Nil
44.	54	F	unknown	112	0.9	0.9	3.6	38	39	65	140	4.0	3	12	+	1-2
45.	51	M	viper	400	0.7	0.9	3.2	43	35	60	136	4.6	3	7	+	2-3
46.	45	M	viper	650	1.0	1.0	3.8	39	40	57	148	4.2	4	9	+	1-2
47.	53	M	viper	650	0.8	0.8	3.9	40	35	78	141	3.8	4	11	+	2-3
48.	55	F	unknown	114	1.1	1.1	3.9	34	34	93	145	4.1	4	11	Trace	Nil
49.	51	F	viper	635	0.9	0.9	3.4	40	41	120	147	4.5	4	9	++	1-2
50.	52	F	unknown	544	0.8	0.8	2.9	33	37	82	138	4.3	3	8	+	1-2

CONTROL

SL. No	Age	Sex	Types of Snake	Plasma NGAL ng/mL	Sr. Creatinine [mg/dL]			Blood Urea [mg/dL]			Electrolytes meq/L		BT mt	CT mt	Urine Examination	
					Day 1	Day 2	Day ≥3	Day 1	Day 2	Day ≥3	Na	K			A1b	RBC
1.	23	M	unknown	42	0.9	1.1	0.9	33	33	30	143	3.8	4	6	Nil	Nil
2.	34	M	unknown	9	1	1.1	1.1	40	41	35	138	4.0	6	7	Nil	Nil
3.	37	M	unknown	14	1.1	1.1	1.1	37	37	28	136	3.5	3	6	Nil	Nil
4.	34	F	unknown	18	0.6	0.8	0.8	36	36	25	147	4.2	6	6	Nil	Nil
5.	47	F	unknown	38	1.1	1	1.1	34	34	38	141	4.7	8	6	Nil	Nil
6.	45	M	unknown	14	0.9	1	1	35	35	36	138	3.7	5	7	Nil	0-1
7.	40	M	unknown	54	0.7	0.9	0.9	36	36	32	142	4.4	4	8	Nil	Nil
8.	29	2	unknown	28	1	1	1	28	29	36	140	4.7	3	6	Nil	Nil
9.	60	M	unknown	30	0.9	1.1	1.1	34	34	42	148	4.5	3	7	Nil	Nil
10.	20	M	unknown	33	0.8	1	0.8	32	32	25	142	3.7	4	7	Nil	Nil
11.	23	M	unknown	36	0.7	0.9	0.8	29	28	26	138	4.7	6	6	Nil	Nil
12.	21	F	unknown	39	0.9	1	0.9	34	35	29	137	3.5	7	9	Trace	Nil
13.	31	M	unknown	42	0.9	1	1	35	35	38	148	3.3	8	8	Nil	Nil
14.	30	M	unknown	35	0.9	1	0.9	38	38	34	145	4.2	5	7	Nil	Nil
15.	32	F	unknown	19	0.8	1	0.8	34	35	32	137	4.4	4	6	+	Nil
16.	33	F	unknown	33	1.1	1.1	1.1	40	40	36	141	4.0	4	7	Nil	Nil
17.	30	F	unknown	26	0.9	0.9	1.0	37	38	23	138	3.5	5	6	Nil	Nil
18.	37	F	unknown	15	1.1	1.1	1.1	38	38	33	140	3.7	3	8	Nil	Nil
19.	44	F	unknown	23	0.7	0.7	0.7	37	37	26	142	4.2	7	8	Nil	0-1
20.	46	F	viper	37	0.6	0.8	0.7	39	40	27	136	3.8	6	7	Nil	Nil
21.	51	M	unknown	48	0.8	0.8	0.8	32	32	42	147	4.0	8	6	Nil	1-2
22.	44	M	unknown	24	0.9	1.0	0.9	39	40	46	136	4.2	7	7	Nil	Nil
23.	45	M	unknown	44	1.0	1.1	1.0	40	40	38	141	3.8	5	8	Nil	Nil
24.	49	M	unknown	32	0.8	0.9	0.9	37	37	34	142	3.7	6	9	Nil	Nil
25.	43	F	unknown	24	1.1	1.0	1.1	40	40	32	140	4.5	7	9	Nil	Nil

SL. No	Age	Sex	Types of Snake	Plasma NGAL ng/mL	Sr.Creatinine [mg/dL]			Blood Urea [mg/dL]			Electrolytes		BT	CT	Urine Examination	
					Day 1	Day 2	Day ≥3	Day 1	Day 2	Day ≥3	Na	K			A16	RBC
26.	57	M	unknown	19	1	1	1.1	33	33	38	135	3.8	6	8	Nil	0-1
27.	52	M	unknown	35	1.0	1.0	0.9	39	39	38	137	4.2	7	7	Trace	Nil
28.	25	M	unknown	29	1	1	1.1	28	29	36	139	4.0	5	8	Nil	0-1
29.	23	M	viper	32	0.8	1.0	1.0	34	34	40	132	3.8	6	8	Nil	Nil
30.	35	M	unknown	38	0.8	1.1	0.8	33	33	23	138	4.0	7	7	Nil	0-1
31.	26	M	unknown	12	0.7	0.9	0.7	36	36	34	141	4.2	8	9	Nil	Nil
32.	42	F	unknown	24	0.9	0.9	0.9	35	35	32	136	3.3	9	7	Nil	Nil
33.	41	M	unknown	38	1.0	1.1	1.1	36	36	36	130	4.7	6	7	Nil	Nil
34.	44	F	unknown	37	1.1	1.1	1.1	41	41	25	147	4.0	7	7	Nil	0-1
35.	40	M	unknown	54	0.7	0.9	0.9	36	36	37	141	3.5	7	8	Nil	0-1
36.	50	M	unknown	52	0.9	0.9	1.0	40	40	37	145	4.0	8	9	Nil	Nil
37.	22	M	unknown	16	0.9	1.1	0.8	39	39	24	134	4.1	9	6	Nil	Nil
38.	27	F	unknown	20	0.7	0.9	0.8	32	33	32	137	3.8	9	7	Nil	Nil
39.	31	M	unknown	24	0.9	1.0	0.8	38	38	38	144	3.7	7	7	Nil	0-1
40.	33	M	unknown	17	1.0	1.1	1.1	34	34	36	142	4.0	8	7	Nil	Nil
41.	48	M	unknown	47	1.0	1.1	1.0	38	38	38	138	4.5	8	8	Nil	Nil
42.	35	F	viper	24	0.8	1.0	0.9	41	41	36	137	3.8	8	9	Nil	0-1
43.	43	M	unknown	19	0.8	0.9	0.8	41	42	36	141	4.8	5	6	Nil	Nil
44.	41	M	unknown	26	1.1	1.1	1.1	39	39	36	147	5.0	4	7	Nil	Nil
45.	53	M	unknown	23	0.7	0.9	0.8	28	28	29	145	4.5	5	9	Nil	Nil
46.	55	F	unknown	22	1.0	1.1	1.0	28	28	27	144	3.8	4	9	Nil	Nil
47.	54	F	unknown	18	0.9	1.0	0.9	35	35	42	147	4.0	5	6	Nil	Nil
48.	58	F	unknown	17	0.9	1.0	0.9	32	32	38	138	4.4	3	7	Nil	Nil
49.	51	F	unknown	34	0.8	0.8	1.0	40	40	33	147	4.3	7	8	Nil	Nil
50.	47	F	unknown	29	1.2	1.1	1.2	37	37	36	143	4.8	5	8	Nil	Nil

PROFORMA

Name :

Age/Sex :

IP No :

Occupation :

Type of Snake : **Time Lapse after snake bite:**

History

Swelling of bitten area: Pain and bleeding from bite site:

Hematuria: Bleeding Gums:

Oliguria/Non Oliguria: Breathlessness:

Clinical Examination:

Local: Vital signs

Cellulitis PR

Skin Changes BP

Tenderness RR

Lymph Node

Systemic Examination:

RS : CVS

ABD CNS

Investigations:

CBC:

PS

BT, CT

Urine Analysis

Blood Sugar:

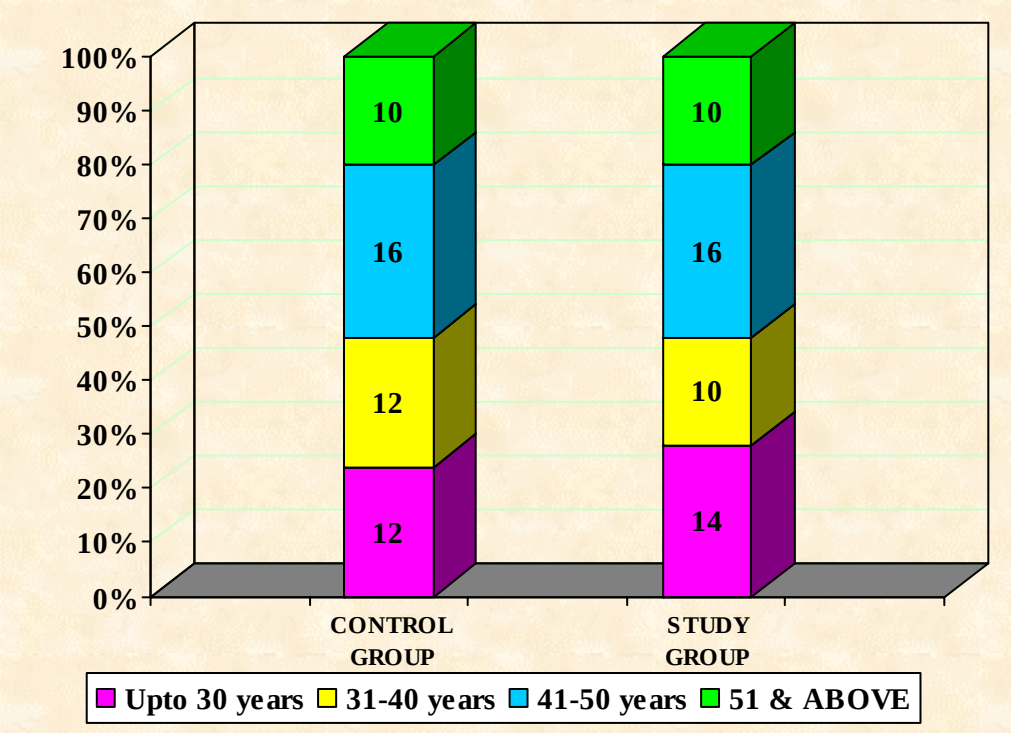
Serum Electrolytes:

Plasma NGAL:

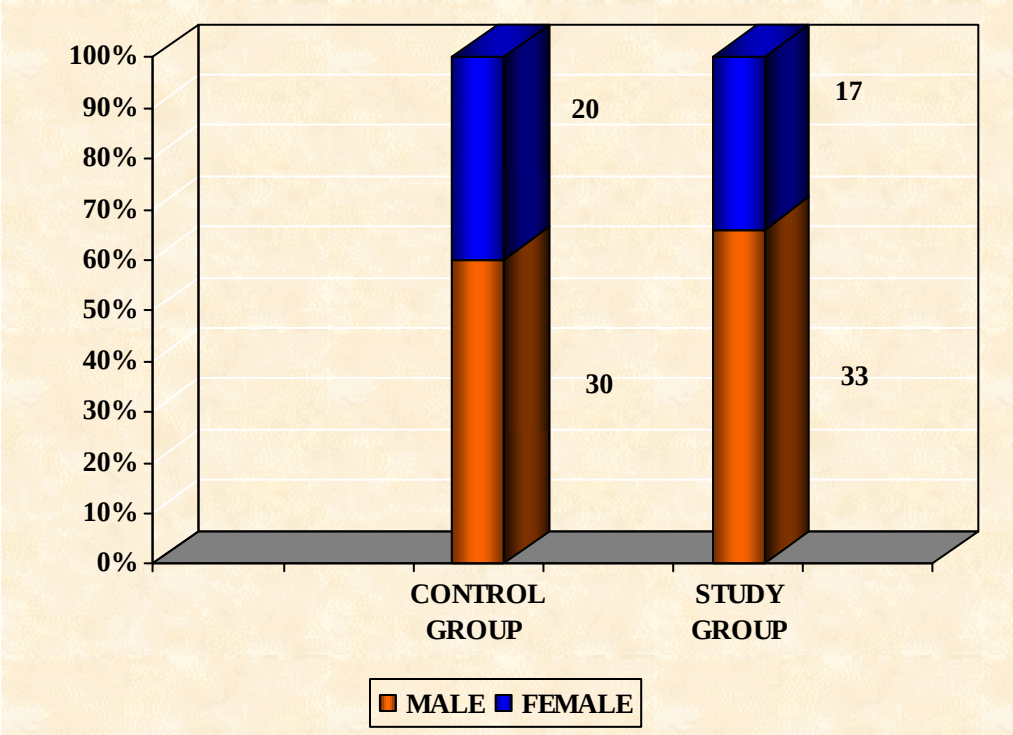
	DAY 1	**DAY 2**	**DAY≥3**
UREA			
CREATININE			

USG ABDOMEN:

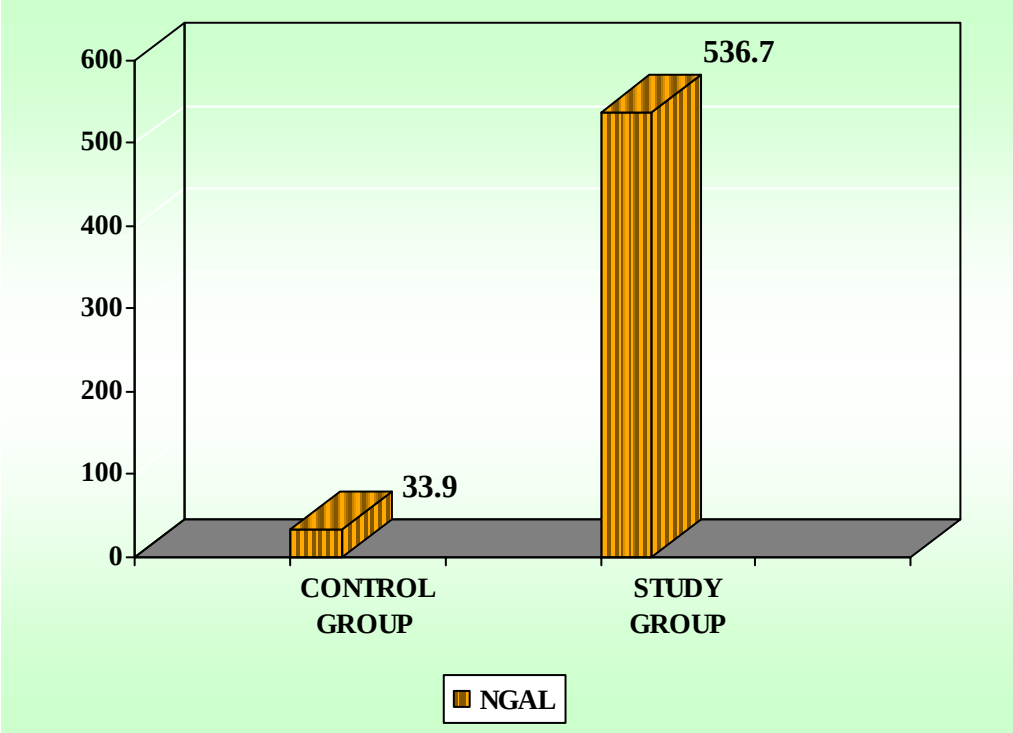
AGE DISTRIBUTION



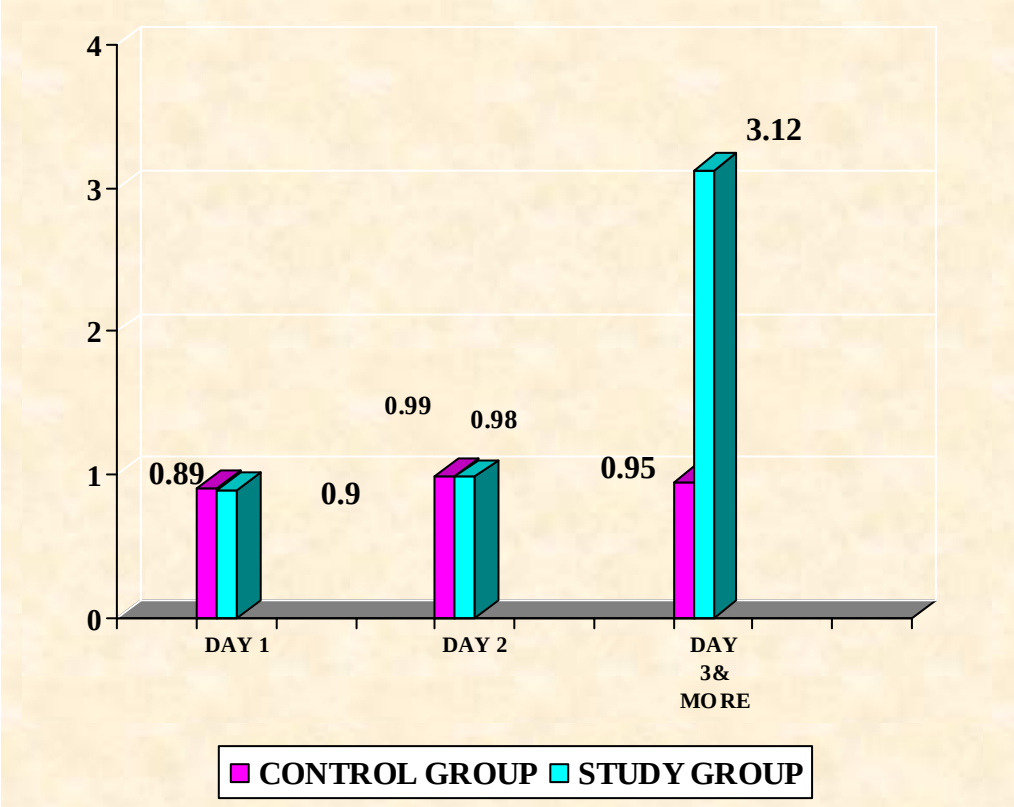
SEX DISTRIBUTION



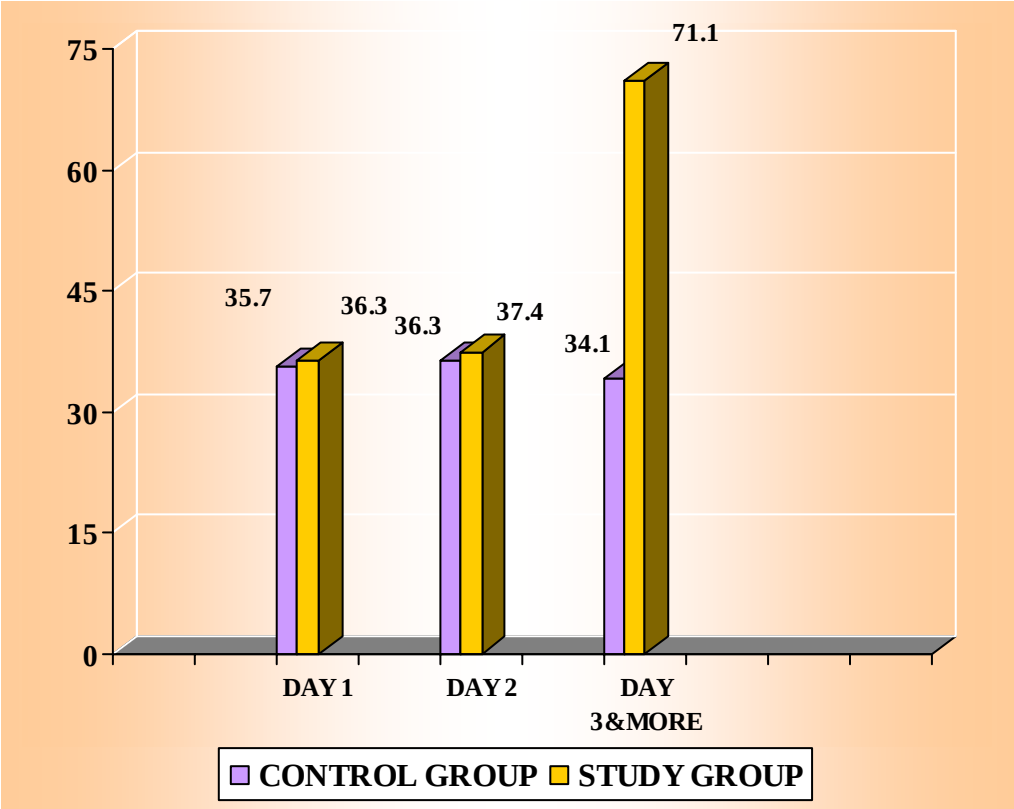
NGAL VALUES


600
500
400
300
200
100
0
536.7
33.9
CONTROL GROUP
STUDY GROUP
NGAL

CREATININE VALUES ON DAY 1,2 AND ≥ 3



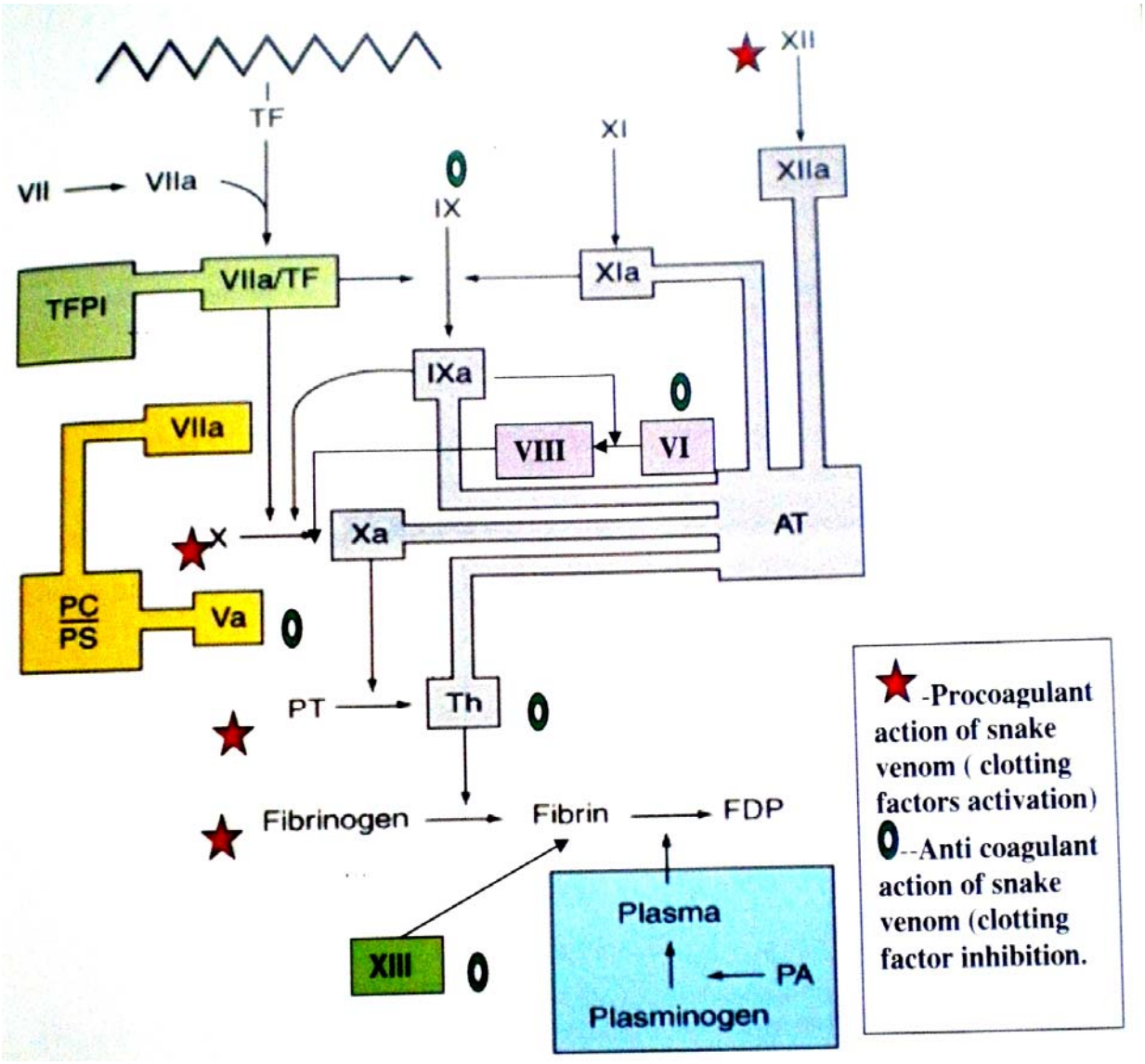
UREA VALUES ON DAY 1,2 AND ≥ 3



SAW SCALED VIPER (SURUTTAI PAMBU)

RUSSELL VIPER(KANNADI VIRIYAN)

SNAKE VENOM AND THE COAGULATION PATHWAY

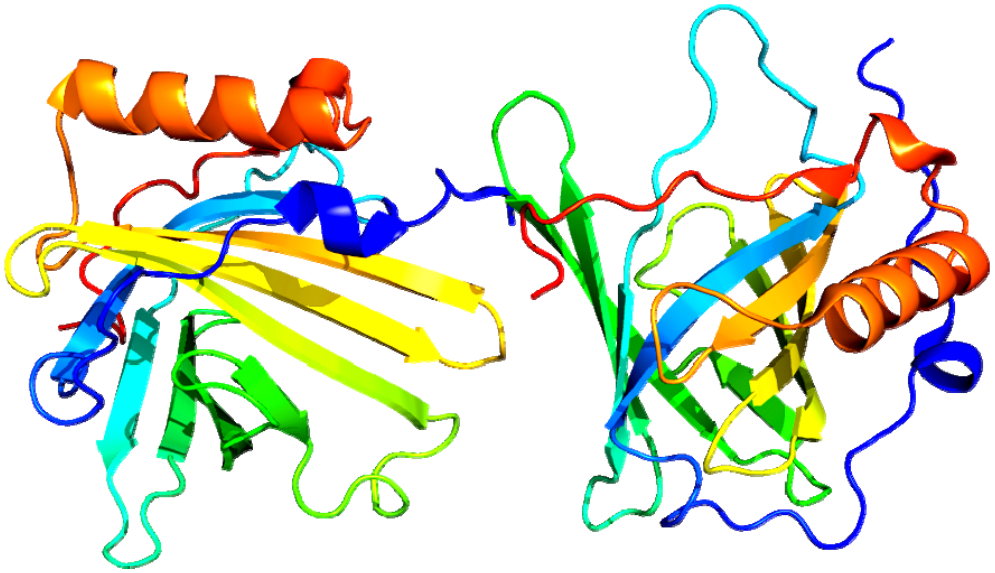


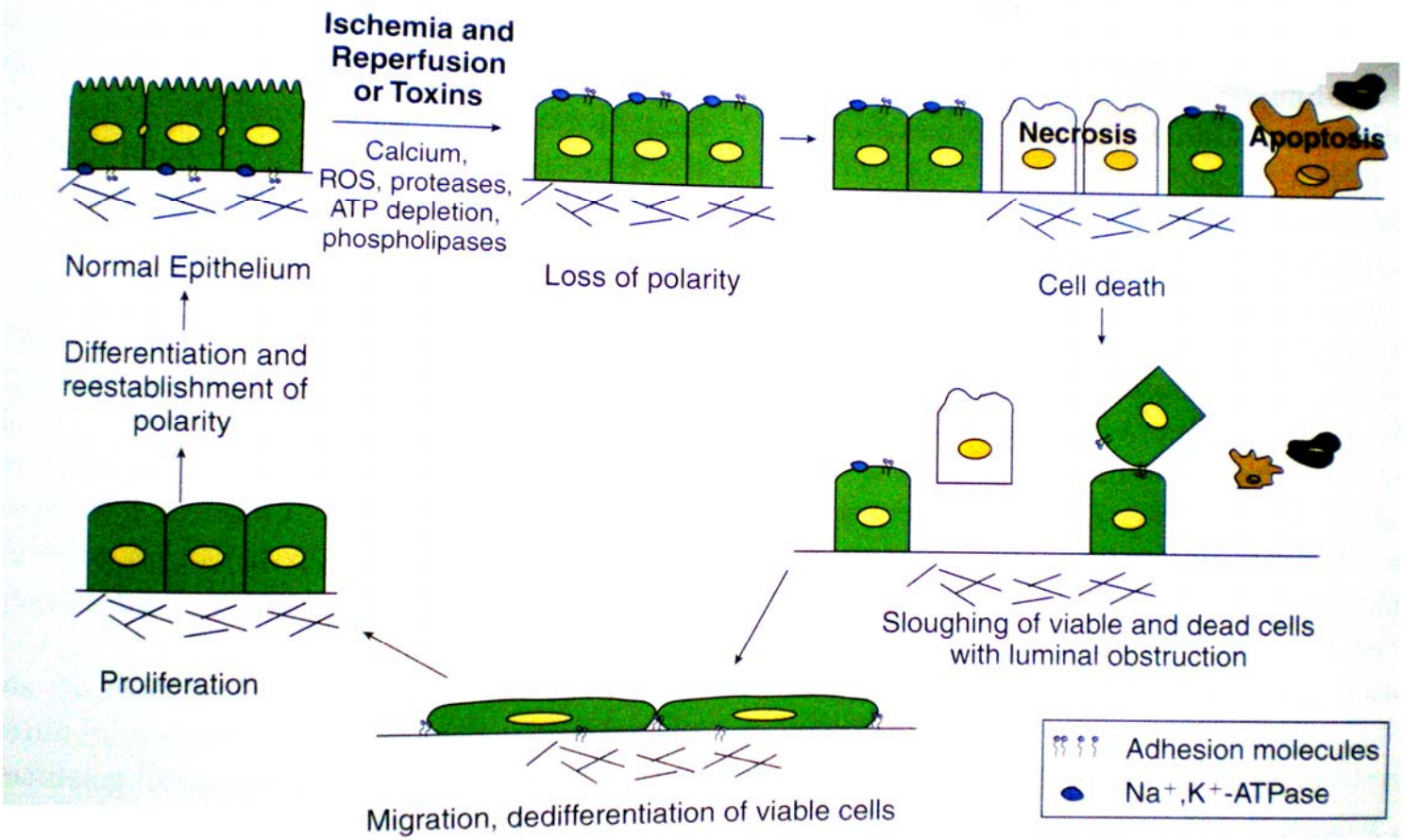
Source:Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo JL Harrison's Principles of Internal Medicine, 17th Edition :http://www.accessmedicine.com

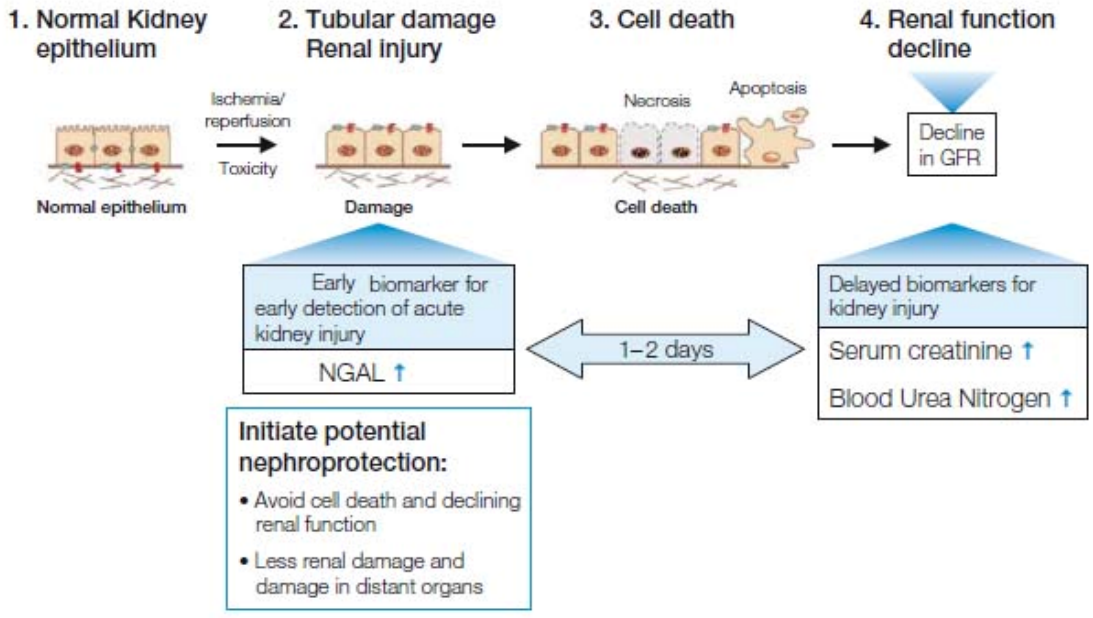
COMMON KRAIT (KATTU VIRIYAN)

COBRA (NAGA PAMBU)

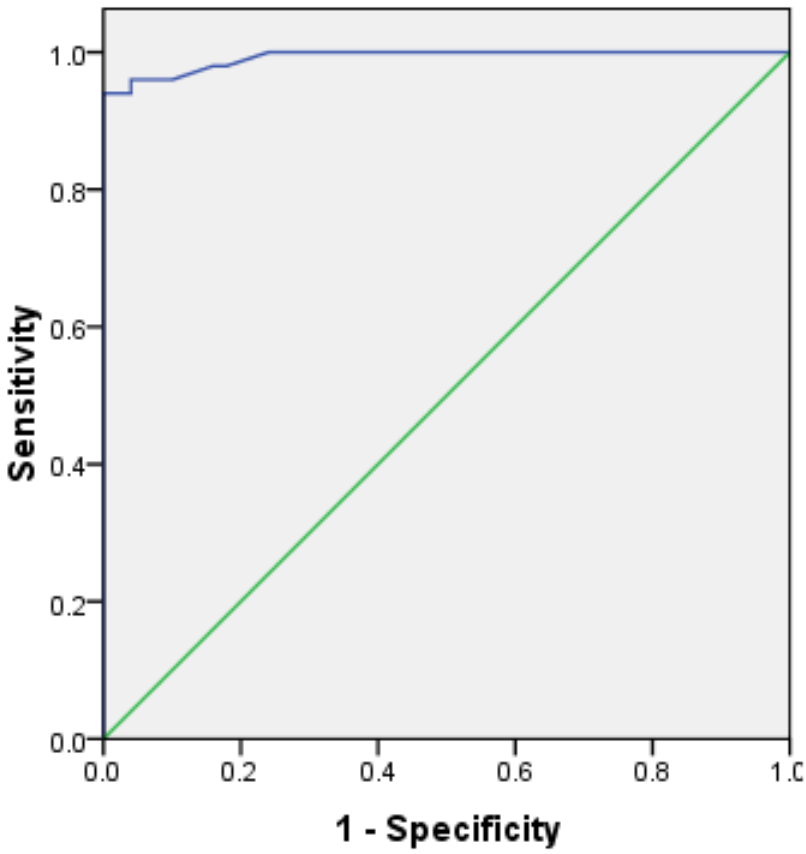
STRUCTURE OF NGAL



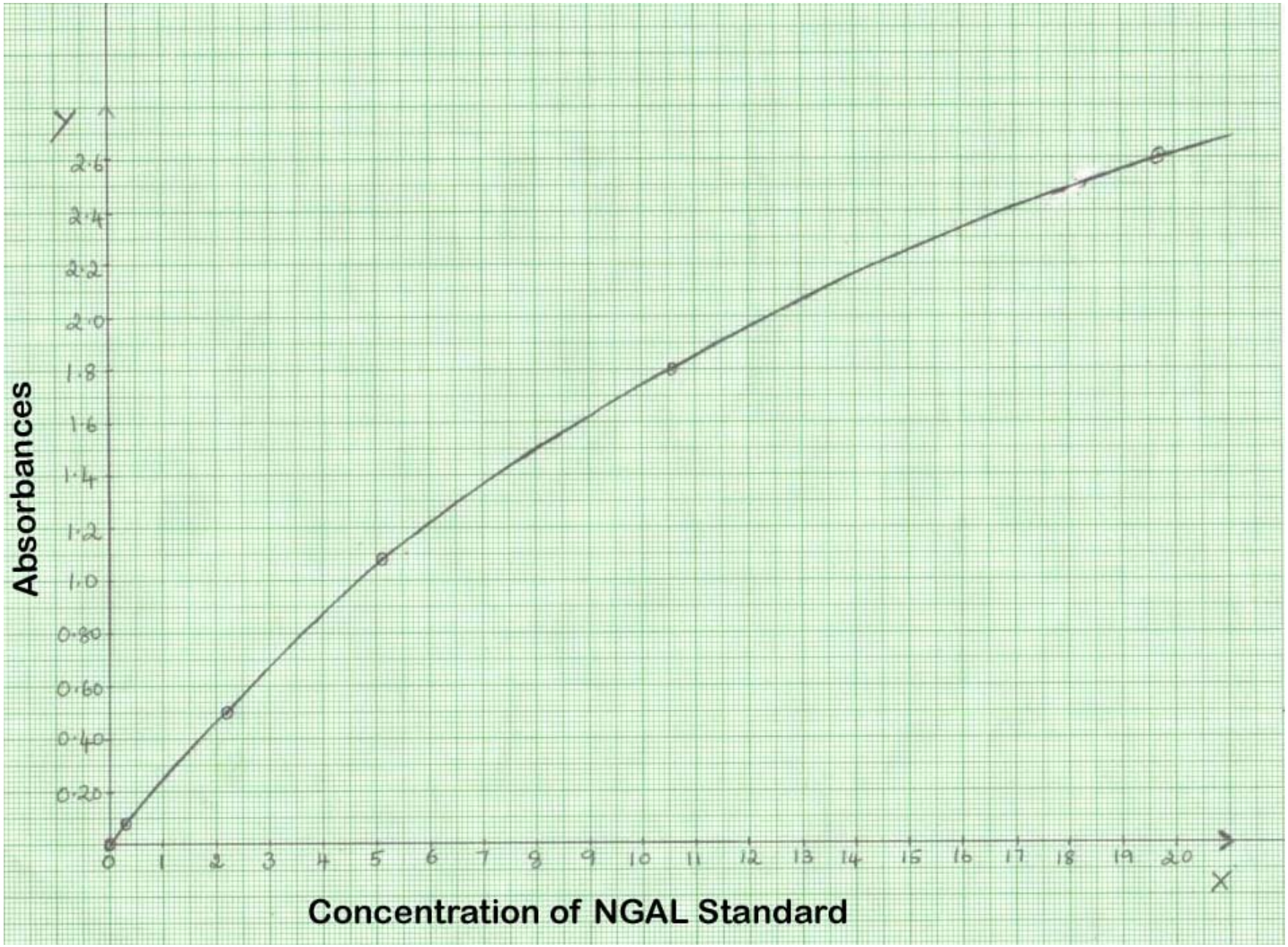

Ischemia and Reperfusion or Toxins
Calcium, ROS, proteases, ATP depletion, phospholipases
Normal Epithelium
Loss of polarity
Necrosis
Apoptosis
Cell death
Differentiation and reestablishment of polarity
Proliferation
Sloughing of viable and dead cells with luminal obstruction
Migration, dedifferentiation of viable cells
Adhesion molecules
Na+,K+-ATPase


1. Normal Kidney epithelium
2. Tubular damage Renal injury
3. Cell death
4. Renal function decline
Ischemia/ reperfusion
Toxicity
Necrosis
Apoptosis
Decline in GFR
Normal epithelium
Damage
Cell death
Early biomarker for early detection of acute kidney injury
NGAL ↑
1–2 days
Delayed biomarkers for kidney injury
Serum creatinine ↑
Blood Urea Nitrogen ↑
Initiate potential nephroprotection:
• Avoid cell death and declining renal function
• Less renal damage and damage in distant organs

ROC curve of NGAL



Diagonal segments are produced by ties.


Absorbances
Concentration of NGAL Standard
Y
X
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·80
0·60
0·40
0·20
0
1
2
3
4
5
6
7
8
9
10
11
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BIBLIOGRAPHY

1. Johy feehally, Jurgen, Floege, Richard J. Johnson. COMPREHENSIVE CLINICAL NEPHROLOGY, 3rd edition, Mosby publishers. Page :7
2. White J. Bites and Stings from venomous animals; a global. Ther Drug Monit 2000; 22: 65-8
3. Warrell DA. Injuries, envenoming, Poisoning and allergic reactions caused by animal in: warrell DA, Cox TN, Firth J.D, Benz J. Jr. editors. OXFORD TEXTBOOK OF MEDICINE OXFORD: Oxford University Press: 2003 p 923-45.
4. Anthony S fauci et- al; Mc Graw Hill ; HARRISON'S PRINCIPLE OF INTERNAL MEDICINE, 17 th edition, Medical Publishers pg:1753
5. Sachin S. Soni Claudio Ronco Nevin Katz et-al; Early diagnosis of Acute Kidney Injury: The promise of Novel Biomarkers:July 8, 2009.
6. Devarajan P. Emerging biomarker discovery in acute kidney injury. Contrib Nephrol 2007; 203-12
7. SELDIN AND GIEBISCH'S THE KIDNEY physiology and pathophysiology; fourth edition: edited by Robert J. Alpern/ Steven C. Hebert volume: Academic press/ Elsevier Publications.
8. Philip E. Snake bite and Scorpion sting. In Srivastava RN editor Paediatric & Neonatal emergency care. Jaypee Brother; New Delhi: 1994.P227-34.
9. David A warrell. WHO/SEARO. Guidelines for the clinical management of Snake Bite in the South-East Asia J Trop Med Pub health; 2005: 1-67.

10. Kulkarni M. Ances. S> Snake venom Poisons, experience with 633 pt. – Indian Paed; 31: 1239-43.
11. WhitakerR; Common Indian Snakes. A field guide. New Delhi. Mac Millan 1978.
12. PRADEEP BAMBERG. API textbook of Medicine. 8th edition: Volume 2: 2008 Pg.No. 1570-1620
13. Warrell D. Animal Toxins. In: Goodon. C. Cook. Manson's Tropical Disease, W.B. Saunder Company Ltd. 1996: 465-515.
14. Chugh KS. Snake-bite induced aute renal failure in India. Kidney Int 1989; 35:891-907.
15. Mittal B.V.Acute renal failure following poisonous snake bite.J. Post Graduate Med: 1994: 40: 123.
16. Dr.K.S. NARAYANA REDDY. Synopsis of Forensic Medicine and Toxicology. 15th edition 2001: Pg. No. 264:270.
17. Wikepedia encyclopedia. November 2008, checklist of the snakes of Srilanka at the Srilanka wildlife conservation society 15,Aug2007.
18. OXFORD TEXTBOOK OF CLINICAL NEPHROLOGY: 3rd edition: edited by Alex.M. Davison. J. Stewart Cameron, Jean Pierre Grunfeld, claudio Ponticelli, Eberhard Ritz, Christopher.G. Winearls, Charles Van Ypersele.
19. Otten EJ. Venomous animal injuries; In: Rosen P.BarlinR, editors. Med comments and clinical practices. Newyork: Mosby:1998 Emergency p924-40.

20. Kohli HS, Sakhuja V. Snake bites and ARF. Saudi J. Kidney Dis. Transpl. 2003; 14: 165-76.

21. Reid HA, Theakston RD. The management of snake bite. Bull WHO 1983; 61:885-95.

22. Greenwood BM, Warrell DA, Davidson NM, Ormerod LD, Reid HA. Immunodiagnosis of snake bite. Br Med J 1974; 4:743-5.

23. Aung W, Kyaw KP, Hla B, Aye SS, Naing SP, Kyaw A, Swe TN Renal Involvement in Russel's viper bite patients without Disseminated Intravascular Coagulation. Trans R Trop Med Hyg 1998;92:322-4.

24. Ratcliffe PJ, Pudrittayakamee S, Ledingham JG, Warrell DA. Direct Nephrotoxicity of Russell's viper venom demonstrated in the isolated Perfused rat kidney. AM J Trop Med Hyg 1989; 40:312-9.

25. J.Anatha Padbanathan. Snake Bite Apicon- 91 API's Medicine Up Date.

26. Seedat TK, Reddy J, Edigton DA. Acute renal failure due to proliferative Pg 74- 79 Nephritis from snakebite poisoning. Nephron 1974;13:455-63.

27. Reid HA. Myoglobinuria and sea-smoke bite poisoning. Br Med j 1961;1:1284-9

28. Harding KA, Welch KR. Venomous Snakes of the world. Acute renal failure following snake bite. Am J Trop Med Hyg 1975;24:692-7

29. Minton SA (ed): Snake Venoms and Envenomation. New York, Marcel Dekker Inc, 1971.

30. Willinger CC, Thamaree S, Schramek H, Gstraunthaler G, Pfaller W. In vitro nephrotoxicity of Russell's viper venom. Kidney Int 1995;45:518-28.

31. Bicher HI, Roth M, Gitter S. Neurotoxic activity of Vipera palestinae Pharmacol Exp. 1996;14:349-59.

32. Peiris OA, Wimalaratne KD, Nimalasuriya A. Exchange transfusions in the treatment of Russell's viper bite Postgrad Med J 1996;45:627-9.

33. Chugh KS, Pal Y, Chakravarty RN, et al. Acute renal failure following poisonous snake bite. AM J Kidney Dis 1984;4:30-8.

34. Jeyarajah R.Russell's viper bite in Sri Lanka. A study of 22 cases. Am J Trop Med Hyg 1984;33:506-1

35. Date A, Pulimood R, Jacob CK, Kirubakaran MG, Shastry JC. Haemolytic uraemic Syndrome complicating snake bite. Nephron 1986;42:89-90.

36. Chugh KS and Sakhuja V. Renal disease caused by snake Venom. Handbook of Natural Toxins Tu AT (ed) New York marcel Dekker Inc 1991;471-93.

37. Kahn MJ, Hemker HC. Studies on blood coagulation factor V.Changes of molecular weight accompanying activation of factor V by thrombin and the procoagulant protein of Russell's Viper venom. Thromb Diath Haemorrh 1972;29:25-32.

38. Kisiel W, Hermo W, hermodson MA. Davie EW. Factor X-activating enzyme from Russell's viper venom: isolation and characterization. Biochemistry 1976;15:4901-6.

39. Rosing J, Tans G. Structural and functional properties of snake venom prothrombin activators. Toxicon 1992;30:1515-27.

40. Rosing J, Tans G. Structural and functional properties of snake venom prothrombin activators. Toxicon 1992;30:1515-27.

41. Ouyang C, Teng CM, Huang TF, Characterisation of snake venom components acting on blood coagulation and platelet function. Toxicon 1992;30:945-66.

42. Markland FSJr. Snake venoms. Drugs 1997; 54(s3):1-10.

43. Sitprija V, Benyajati C, Boonpucknavig V. Further observations of renal insufficiency in snakebite. Nephron 1974;13:396-403.

44. Lakier JB, Fritz VU. Consumptive coagulopathy caused by a bloomslang bite. S Afr Med J 1969;43:1052-5.

45. Date A, Shastry JC. Renal ultrastructure in acute tubular necrosis following Russell's viper envenomation. J Pathol 1982;137:225-41.

46. Kjeldsen L, johnsen AH, sengelov H, Borregaard N (1993) Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 268:10425 -10432.

47. Xu SY, Carlson M, Engstrom A, Garcia R, Peterson CG, Venge P (1994) Purification and characteristation of a human neutrophil lipocalin (HNL) from the secondary granules of human neutrophils. Scand J Clin Lab Invest 54:365-376.

48. Mori K, Lee HT , Rappoport D , Drexler IR , Foster K, Yang J, Schidt –Ott KM , Chen X, Li JY, Weiss S, Mishra J , Cheema FH, Markowitz G,

Suganami T, Sawai K, Mukoyama M, Kunis C, D' Agati V, Devarajan P, Barasch J (2005) Endocytic delivery of lipocalin- siderophore- iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 115:610-621.

49. Mishra J, Ma Q, Prada A, et al, Identification of neutrophil gelatinase - associated lipocalin as a novel early urinary biomarker for renal injury. J Am Soc Nephrol 14:2534-2543.

50. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, Mori K, Barasch J, Devarajan P (2005) Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury. Lancet 365(9466):1231-1238.

51. Schmidt-Ott KM, Mori K, Kalandadze A, Li JY, Paragas N, Nicholas T, Devarajan P, Barasch J. NGAL-mediated iron traffic in kidney epithelia. Curr Opin Nephrol Hypertens. 2006;15:442-449.

52. Bennett M.Dent CL,Devarajan P et al.Urine NGAL predicts severity of acute kidney injury,a prospective study.Clin J Am Soc Nephrol 2008;3:665-673.

53. Chirag R Parikh, Jonathan C Lu, steven G Coca, Prasad Devarajan, Tubular proteinuria in acute kidney injury: a critical evaluation of current status and future promise. Annals of Clinical Biochemistry July 2010; Volume 47; Part 4:301-312.

54. Greenberg, cheung, Coffman, Falk Jennette. PRIMER ON KIDNEY DISEASES.5th edition.Saunders ELSEVIER publishers.32;270-283.

55. Parikh CR, Jani A, Mishra J, et al. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant 2006;6:1639-45.

56. Nickolas TL, O'Rourke MJ, Yang J, et al. Sensitivity and specificity of a single emergency department measurement of urinary NGAL for diagnosing acute kidney injury. Ann Int Med 2008;148:810-9.

57. Tuladhar SM, Puntmann VO, et al. Rapid detection of acute kidney injury by plasma and urinary neutrophil gelatinase-associated lipocalin . J Cardiovasc Pharmacol 2009;53:261-6.

58. Uttenthal O: NGAL: a marker for the distressed kidney? Clin Lab Internat 2005;29:39-41.

59. Nguyen MT, Devarajan P: Biomarkers for the early detection of acute kidney injury. Pediatr Nephrol 2008;23:2151-2157.

60. Haase M, Bellomo R, Devarajan P,et al; Accuracy of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diagnosis and Prognosis in Acute Kidney Injury: A systematic Review and Meta-analysis.Am J kidney Dis 2009 .

61. Syed Moied Ahmed ,Mohib Ahmed, et al. Emergency Treatment of a snake bite; pearls from literature. J Emergencies, Trauma and Shock; 1:2; Jul-Dec 2008.

62. Dr. med. Michael Haase, Dept of Nephrology and Intensive Care Medicine Charite University Medicine, Campus Virchow Klinikum, Berlin, Germany- NGAL to predict Acute Kidney Injury- Potential applications and limitations.